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(54) Title
RECOMBINANT VACCINIA VIRUS FOR PREVENTION OF DISEASE CAUSED BY FLAVIVIRUS

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(57) The present invention is related to the construction of recombinant vaccinia viruses. More particularly, the present invention is related to the construction of recombinant vaccinia viruses which are useful for the preparation of a vaccine for the prevention of diseases caused by flaviviruses, such as dengue virus, Japanese B encephalitis virus and tick-borne encephalitis virus.

Claim

1. A recombinant vaccinia virus selected from the group consisting of V(C-M-E-NS1-NS2_A), V(C-M-E), V(E), V(RSV G-E), V(FLU HA-E), and V(NS1-NS2_A), wherein the viral proteins are obtained from a flavivirus.

2. A recombinant vaccinia virus consisting essentially of the coding sequence for C, M, E, NS1, and NS2_A proteins, wherein the viral proteins are obtained from a flavivirus and 85 percent of the carboxyl terminal sequence of NS2_A protein is deleted.

7. A pharmaceutical composition, comprising an immunogenic amount of the virus of claim 1 in a pharmaceutically acceptable carrier.

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10. A method for inducing protective immunity against a flavivirus disease, comprising inoculating a host acceptable to a flavivirus with an immunogenic amount of the virus of claim 2 in at least a single dose.

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(21) International Application Number: PCT/US89/03589 (22) International Filing Date: 21 August 1989 (21.08.89) (30) Priority data: 239,205 20 August 1988 (20.08.88) US (71) Applicant: THE UNITED STATES OF AMERICA, represented by THE SECRETARY, UNITED STATES DEPARTMENT OF COMMERCE [US/US]; Washington, DC 20231 (US). (72) Inventors: LAI, Ching-Juh ; 7353 Heatherhill Ct., Bethesda, MD 20817 (US). BRAY, Michael, P. ; 5019 Acacia Avenue, Bethesda, MD 20814 (US). ZHAO, Bangti ; 4932 Battery Lane, ,6, Bethesda, MD 20814 (US). CHANOCK, Robert, M. ; 7001 Longwood Drive, Bethesda, MD 20817 (US).		(74) Agents: OLIFF, James, A. et al.; Oliff & Berridge, 277 S. Washington Street, Alexandria, VA 22314 (US). (81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent). Published <i>With international search report.</i>
(54) Title: RECOMBINANT VACCINIA VIRUS FOR PREVENTION OF DISEASE CAUSED BY FLAVIVIRUS (57) Abstract A recombinant vaccinia virus contains complete coding sequence for the expression of flavivirus antigenic proteins. A vaccine against flavivirus disease is also provided.		

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RECOMBINANT VACCINIA VIRUS FOR PREVENTION
OF DISEASE CAUSED BY FLAVIVIRUSTechnical Field

5 The present invention is related to the construction of recombinant vaccinia viruses. More particularly, the present invention is related to the construction of recombinant vaccinia viruses which are useful for the preparation of a vaccine for the prevention of diseases caused by flaviviruses, such as dengue virus, Japanese B encephalitis virus and tick-borne encephalitis virus.

Background of the Invention

15 Certain diseases of public health concern, such as dengue disease and encephalitis of certain types are known to be caused by viruses belonging to the flavivirus family. It has been estimated that up to 100 million illnesses occur every year in tropical areas of the world due to dengue virus alone. However, there is no effective and specific immunoprophylactic measure to control such flavivirus diseases.

SUMMARY OF THE INVENTION

25 It is, therefore, an object of the present invention to provide a recombinant vaccinia virus which induces an immune response against a specific flavivirus disease in a host infected with said recombinant vaccinia virus.

Other objects and advantages of the present invention will become evident from the following detailed description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

30 These and other objects, features and many of the attendant advantages of the invention will be better understood upon a reading of the following detailed description when considered in connection with the accompanying drawings wherein:

35 Figure 1 shows schematic construction of a dengue virus-vaccinia virus recombinant plasmid. The intermediate cloning vector psc11 contains interrupted

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thymidine kinase gene sequences (TK^R and TK^L) and a bacterial β -galactosidase gene (LacZ) under the transcription control of the P11 vaccinia virus promoter. The vector DNA was partially digested with BamHI to open the BamHI site downstream of the P7.5 promoter for insertion of the 4.1 kilobase (Kb) fragment of dengue virus DNA located at the 5' end of the viral genome. This fragment of dengue cDNA contains the coding region for the three dengue structural proteins [capsid (C), pre-membrane (pre-M), and envelope glycoprotein (E)] plus the first two downstream non-structural proteins NS1 and NS2a. Recombinant plasmid containing the dengue virus DNA insert in the sense transcription orientation was selected and used for construction of the recombinant vaccinia virus.

Figures 2A and 2B demonstrate the identification of dengue virus proteins synthesized by the recombinant vaccinia virus. (Figure 2A:) Immunoprecipitation of [³⁵S]-methionine-labeled lysate from recombinant vaccinia virus-infected cells (multiplicity of infection, 10 PFU per cell) was carried out with one of the following specific antibodies: monoclonal antibody 1H10, specific for the envelope glycoprotein (E); rabbit antibodies prepared against dengue virus type 2 nonstructural protein 1(NS₁); monoclonal antibody 5C9 specific for the membrane glycoprotein precursor (preM); and polyvalent antibodies (P). The labeled precipitates were analyzed on sodium dodecyl sulfate-12% polyacrylamide gels. Also shown are the labeled dengue virus protein markers (D) obtained by immunoprecipitation with polyvalent antibodies of [³⁵S]-methionine-labeled lysate from dengue virus-infected CV-1 cells. (Figure 2B:) Labeled immunoprecipitates prepared as described above were analyzed by digestion with endoglycosidase H (endo H) and by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Numbers at the left of the gels indicate protein sizes in kilodaltons.

DETAILED DESCRIPTION OF THE INVENTION

The above and various other objects and advantages of the present invention are achieved by a recombinant vaccinia virus containing complete coding sequence for the expression of the major specific protective flavivirus antigens in cells infected with said recombinant vaccinia virus, said antigens inducing protective immunity against infection by said flavivirus in a susceptible host.

Accordingly, the present invention provides a recombinant vaccinia virus selected from the group consisting V(C-M-E-NS1-NS2a), V(C-M-E), V(E), V(RSV G-E), V(FLU HA-E), and V(NS1-NS2_A), wherein the viral proteins are obtained from a flavivirus.

The present invention further provides a recombinant vaccinia virus consisting essentially of the coding sequence for C, M, E, NS1, and NS2_a proteins, wherein the viral proteins are obtained from a flavivirus and 85 percent of the carboxyl terminal sequence of NS2_A protein is deleted.

The present invention also provides a pharmaceutical composition, comprising an immunogenic amount of the above virus in a pharmaceutically acceptable carrier.

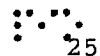
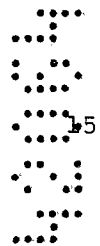
The present invention also provides a method for inducing protective immunity against a flavivirus disease, comprising innoculating a host acceptable to flavivirus with an immunogenic amount of the above virus in at least a single dose.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned hereunder are incorporated herein by reference. Unless mentioned otherwise, the techniques employed herein are standard methodologies well known to one of ordinary skill in the art.

Like other members of the flavivirus family,



extracellular dengue virus has a relatively simple structure. Virions contain only three virus-coded proteins, designated capsid (C) protein, membrane (M) protein, and envelope (E) glycoprotein. Intracellular virus, which is also infectious, lacks M but contains another glycoprotein, preM, from which M is derived by cleavage. Both C and M are internal proteins. The surface envelope glycoprotein is the major site responsible for neutralization of infectivity by specific antibodies. The envelope glycoprotein also exhibits hemagglutinating activity and is responsible for adsorption to the cell surface. Several nonstructural proteins have also been identified in dengue virus-infected cells. Among them is nonstructural protein NS1, which is a glycoprotein and described as a soluble complement-fixing



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antigen. It is believed that NS1 plays an important role in mediating immunity, since the analogous NS1 glycoprotein of yellow fever virus has been shown to be a protective antigen in mice and in primates. Furthermore, the
5 NS1 of dengue type 2 virus has been shown to be a protective antigen.

The dengue type 4 virus genome consists of a molecule of positive-stranded RNA 10,644 nucleotides in length. A full-length cDNA copy of the dengue type 4
10 virus has been prepared, and its complete nucleotide sequence determined. From such studies, it has been estimated that 96% of the dengue virus genome codes for a polyprotein which is cleaved by specific protease(s) to generate individual viral proteins. The three structural
15 proteins, C-M-E, are located at the amino terminus, while nonstructural proteins NS1-NS2a-NS2b-NS3-NS4a-NS4b-NS5 are at the carboxy-terminus. It must be emphasized that because dengue virus gene expression involves proteolytic cleavage of the polyprotein, it cannot be assumed a
20 priori that the expression of protective antigens such as E and NS1 from their cloned DNA sequences, would be useful in immunoprophylaxis. Such determination can be made only through actual testing. The construction of a vaccinia flavivirus recombinant is now illustrated by
25 employing dengue virus genes as an example.

Vaccinia virus was used as a vector for construction of a live recombinant virus expressing dengue viral genes. The BglIII DNA fragment (4,041 base pairs, nucleotides 88 to 4128) from the 5' terminus of dengue
30 type 4 virus cDNA contains the coding region for the three structural proteins as well as nonstructural proteins NS1 and NS2a. This fragment was excised from the full-length dengue virus DNA copy and was inserted into the PSC11 vaccinia intermediate vector. The dengue virus
35 DNA sequence was inserted in the BamHI site immediately downstream of the vaccinia P7.5 early-late promoter (Figure 1). In this construct, the dengue virus coding

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sequence was placed under the transcriptional control of the vaccinia virus P7.5 early-late promoter. The vector contained a bacterial β -galactosidase gene under the control of the vaccinia virus P11 late promoter, which provided a visual selectable marker. The chimeric genes were flanked by sequences of the vaccinia virus thymidine kinase gene, which directed homologous recombination of dengue virus sequences into the vaccinia virus genome following transfection of simian CV-1 cells previously infected with wild type vaccinia virus (WR strain). Recombinant vaccinia virus harboring the dengue virus DNA insert was isolated and plaque purified two times on thymidine kinase minus (TK) cells in selective medium. Other dengue cDNA fragments, vide infra, were inserted into a vaccinia recombinant virus by the method just described. Similarly, constructs of other flavivirus-vaccinia recombinants are made by inserting flavivirus cDNA following the procedure described herein for dengue virus.

Dengue virus-specific proteins synthesized during infection with the recombinant virus were initially detected by indirect immunofluorescence. CV-1 cells infected with the recombinant virus exhibited fluorescent-stainable antigens in the cytoplasm when polyvalent dengue type 4 virus hyperimmune mouse antiserum was used, although the intensity of staining was less than that observed in dengue virus-infected cells. Similar immunofluorescence was observed in CV-1 cells infected with the recombinant virus when monoclonal antibody specific to the E glycoprotein was used.

To further identify this and other dengue virus proteins, recombinant virus-infected cells were radio-labeled with [³⁵S]methionine and the cell lysate was prepared for immunoprecipitation by specific antibodies. Analysis of the labeled precipitate on sodium dodecyl sulfate-polyacrylamide gel (Figure 2A) showed that the polyvalent hyperimmune mouse antiserum precipitated three



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major bands with estimated molecular size 20, 40 to 46, and 55 to 60 kilodaltons (kDa), respectively. Minor bands of larger molecular size that were probably non-specific were also observed. When an E glycoprotein-specific monoclonal antibody (1H10) was used, a 55 to 60-kDa band consistent with the molecular size of the E glycoprotein was precipitated. Similarly, dengue type 2 virus NS1-specific antiserum (obtained from Dr. J. Schlesinger) precipitated a 40- to 46-kDa band, which is the predicted size for the NS1 nonstructural glycoprotein. The third major band precipitated by dengue type 4 virus hyperimmune antiserum was approximately 20 kDa which is consistent with the size of the intracellular preM glycoprotein. A similar band was precipitated by preM-specific antibodies (monoclonal antibody 2H2 or 5C9, obtained from Dr. M. K. Gentry of WRAIR).

Thus, in recombinant virus-infected cells, the three glycoproteins encoded in the cloned DNA appeared to be cleaved and modified by glycosylation in a manner similar to that observed during dengue virus infection. This suggests that the dengue viral structural proteins, as well as the NS1 nonstructural protein, were specifically processed by proteolytic cleavage of the polyprotein in the absence of dengue virus functions provided by the distal nonstructural proteins NS2b through NS5. However, as estimated by immunoprecipitation, the amount of dengue preM, E and NS1 glycoproteins produced in recombinant virus infected cells was significantly less than in dengue type 4 virus-infected cells.

The pattern of glycosylation of the glycoproteins produced in recombinant virus-infected cells varied as indicated by their response to endoglycosidase H, which cleaves the mannose rich carbohydrate core. The preM protein band was completely sensitive to endoglycosidase H treatment, yielding a band of 17 kDa, a reduction of 3 kDa in molecular size. On the other hand, a significant portion of the carbohydrate of both the E

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and the NS1 glycoproteins appeared to be resistant to endoglycosidase H digestion.

Each of the dengue virus glycoproteins, i.e., preM, E and NS1, is preceded by a stretch of hydrophobic amino acids which can serve as a signal. This indicates that cellular signalase is probably responsible for proteolytic cleavage. Presumably, the cleavage mechanism which generates the three glycoproteins, also yields the capsid protein, which is located amino terminal to the preM glycoprotein.

As described herein above, initially a recombinant vaccinia virus containing a 4.0 Kb sequence from the 5' terminus of cDNA that codes for the three structural proteins [capsid (C), membrane (M), and envelope (E)] and nonstructural proteins NS1 and NS2a was constructed. Subsequently, another recombinant vaccinia virus expressing only the three structural proteins was constructed by deleting the coding sequence for non-structural proteins. Protein analysis showed that cells infected with the first recombinant virus v(C-M-E-NS1-NS2a) produced authentic pre-membrane (preM), envelope (E), and NS1 glycoproteins as detected by radioimmuno-precipitation using specific antibodies. These dengue proteins showed a glycosylation pattern similar to that found for these proteins produced during dengue virus infection. The second recombinant virus (C-M-E) produced dengue virus preM and E glycoproteins. As expected, NS1 was not synthesized by the second recombinant (Table 2).

The mouse model of dengue encephalitis was employed to evaluate protective immunity induced by these recombinants. Mice were inoculated by the intraperitoneal route with 10^7 plaque forming units (pfu) of recombinant vaccinia virus. Mice were reinoculated with the same dose of recombinant two weeks later. One week after the second inoculation the mice were challenged with 100 LD₅₀ of dengue virus intracerebrally. The animals were then observed for signs of encephalitis and death.

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Ninety-one percent of mice immunized with a control vaccinia recombinant virus expressing the envelope glycoprotein of HIV died after challenge (Tables 1 and 2). In contrast, 96% of mice immunized with the vaccinia (C-M-E-NS1-NS2a) recombinant that expressed the three structural proteins and NS1, survived. Also, all 15 animals immunized with the vaccinia (C-M-E) recombinant virus that expressed only the three structural proteins were completely protected. These results indicate that structural proteins alone are able to induce protective immunity. Since C and M are internal proteins, it is concluded that E is the major antigen responsible for protection conferred by the v(C-M-E) recombinant. This interpretation is supported by the complete resistance to intracerebral challenge induced by immunization with vaccinia recombinants that expressed only the E glycoprotein. Data in Table 1 provide such evidence. A recombinant that expressed E including its N-terminal hydrophobic signal sequence, or a respiratory syncytial virus (RSV) G glycoprotein (amino acids 1 through 70) - E fusion protein or an influenza A virus hemagglutinin N terminal signal peptide - E fusion protein induced complete protection against dengue virus challenge.

The seroresponse of animals immunized with these recombinants was tested by radioimmunoprecipitation of labeled antigens. Each of the animals immunized with the v(C-M-E-NS1-NS2a) recombinant that expressed the three structural proteins and NS1, developed NS1 antibodies, while the amount of antibody to E was low or not detectable. The low level of E antibody response was further confirmed by other serologic tests such as virus neutralization and ELISA. Also, mice infected with the vaccinia recombinant (C-M-E) that expressed only the three structural proteins developed little or no detectable E antibody.

The nonstructural protein NS1 expressed by various vaccinia recombinant viruses was also evaluated

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for its antigenic properties and the most promising products were evaluated for their protective effect in mice. A total of 6 NS1 vaccinia recombinants were studied. These recombinants contained an insert in which: (i) NS1 was preceded by the structural protein sequence (C-M-E), (ii) NS1 was preceded only by its immediate upstream hydrophobic sequence or (iii) NS1 was not preceded by structural genes or its immediate upstream hydrophobic sequence. Each of these inserts terminated with the complete NS2a sequence or the N terminal 15% of the NS2a sequence. The v(C-M-E-NS1-NS2a) and v(NS1-NS2a) recombinants produced authentic NS1, whereas other recombinants that lacked the NS1 N terminal hydrophobic signal or lacked the complete downstream NS2a sequence yielded a NS1 product of different molecular size and/or were not glycosylated as indicated by gel analysis. From these studies it was concluded that the N-terminal signal sequence of NS1 and the complete downstream NS2a sequence are required for proper processing and proteolytic cleavage of NS1.

The above mentioned NS1 recombinants were then evaluated for their prophylactic potential in mouse protection studies as described above. The results indicated that all but one of 28 mice inoculated with vaccinia (C-M-E-NS1-NS2a) recombinant expressing the structural proteins and NS1 survived dengue challenge (Table 1). Significantly, the recombinant which expressed authentic NS1 in the absence of structural proteins, i.e., v(NS1-NS2a), induced complete resistance to lethal challenge with dengue virus (Table 2). Other vaccinia recombinants that expressed abnormal NS1 induced only partial protection. Thus, vaccination with a vaccinia recombinant that expressed (i) an upstream N-terminal RSV G sequence (amino acids 1-70) fused to NS1 or (ii) NS1 plus 15% of NS2a, induced only partial protection.

These tests demonstrate that dengue envelope

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(E) glycoprotein and nonstructural protein NS1 are separate protective antigens, each of which can induce complete resistance to encephalitis in the mouse model of dengue disease. These results now allow the development of vaccinia recombinants expressing E and/or NS1 glycoprotein as vaccines for protection of humans against dengue type 4 virus. Accordingly, a vaccine comprises an immunogenic amount of the recombinant vaccinia virus which induces protective immunity against a specific flavivirus in a host infected with said recombinant vaccinia virus. Conventional pharmaceutically acceptable carrier or vehicles such as non-toxic buffers, physiological saline and the like could be used together with adjuvants and booster inoculations, if necessary. Of course, the vaccine can be administered in a single or multiple dosages as indicated.

It is important to point out here that a vaccinia-dengue type 2 virus recombinant that contained the dengue sequences for C, M, and E controlled by a P11 vaccinia virus promoter was recently reported by Deubel et al, 1988 (J. Gen. Virol. 69:1921-1929) and failed to protect monkeys against dengue. In contrast, the recombinant of the present invention was efficacious in protecting the test animals against viral infection.

Since the genome organization, replication strategy and gene expression of all flaviviruses are similar, recombinant vaccinia viruses having prophylactic property against other flaviviruses are made in a manner similar to the herein illustrated vaccinia-dengue construct.

A deposit of the recombinant virus has been made at the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A., on August 19, 1988 under the accession number VR 2228. The deposit shall be viably maintained, and replaced if it becomes non-viable, for a period of 30 years from the date of the deposit, or for 5 years from the last date of

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request for a sample of the deposit, whichever is longer,
and made available to the public without restriction in
accordance with the provisions of the law. The
Commissioner of Patents and Trademarks, upon request,
5 shall have access to the deposit.

It is understood that the examples and embodi-
ments described herein are for illustrative purposes only
and that various modifications or changes in light there-
of will be suggested to persons skilled in the art and
10 are to be included within the spirit and purview of this
application and the scope of the appended claims.

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TABLE 1
MICE INFECTED WITH VACCINIA RECOMBINANTS EXPRESSING
DENGUE ENVELOPE (E) GLYCOPROTEIN WITH OR WITHOUT
NONSTRUCTURAL NS1 GLYCOPROTEIN DEVELOP
RESISTANCE TO FATAL CHALLENGE WITH DENGUE VIRUS

5

10	VACCINIA RECOM- BINANT EXPRESSING DENGUE PROTEIN(S)	EXPRESSION OF		NO. OF MICE INFECTED (10 ⁷ PFU)	NO. WHICH SURVIVED AN INTRACEREBRAL CHALLENGE WITH 10 ² LD ₅₀ DENGUE TYPE 4 VIRUS
		GLYCO- SYLATED E	AUTHEN- TIC NS1		
	v(C-M-E-NS1-NS2 _A)	YES	YES	28	27 (96%)
	v(C-M-E-NS1-NS2 _A ^Δ)*	YES	NO	15	15 (100%)
	v(C-M-E)	YES	-	19	19 (100%)
	v(E)	YES	-	15	15 (100%)**
15	v(RSV G-E) ⁺	YES	-	15	15 (100%)**
	v(FLU HA-E) ⁺⁺	YES	-	15	15 (100%)
	v(HIV GP160) ⁺⁺⁺	-	-	46	4 (8.7%)

* 85% OF C TERMINAL SEQUENCE OF NS2_A DELETED.

** SURVIVING MICE DEVELOPED MILD SIGNS OF ENCEPHALITIS.

20 + EXPRESSES FUSION PROTEIN OF N TERMINUS OF RSV G (AMINO ACIDS 1-70) PLUS E.

++ EXPRESSES FUSION PROTEIN OF INFLUENZA A VIRUS N TERMINAL SIGNAL PEPTIDE PLUS E.

+++ CONTROL VACCINIA RECOMBINANT EXPRESSING ENVELOPE GLYCOPROTEIN OF
25 HIV.

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TABLE 2
MICE INFECTED WITH VACCINIA RECOMBINANT EXPRESSING ONLY
DENGUE NONSTRUCTURAL PROTEIN NS1 DEVELOP RESISTANCE
TO FATAL CHALLENGE WITH DENGUE VIRUS

5	VACCINIA RECOM- BINANT EXPRESSING INDICATED DENGUE PROTEIN(S)	EXPRESSION OF AUTHENTIC NS1	NO. OF MICE INFECTED (10 ⁷ PFU)	NO. WHICH SURVIVED AN INTRACEREBRAL CHAL- LENGE WITH 10 ² LD ₅₀ DENGUE TYPE 4 VIRUS
10	v(NS1-NS2 _A)	YES	15	15 (100%)
	v(NS1-NS2 _A ^Δ)*	NO	30	20 (67%)
	v(RSV G-NS1) ⁺	NO	15	8 (54%)
	v(HIV GP160) ⁺⁺	-	46	4 (8.7%)

* 85% OF C TERMINAL SEQUENCE OF NS2_A DELETED.

15 + EXPRESSES FUSION PROTEIN OF RSV G (AMINO ACIDS 1-70) PLUS NS1.

++ CONTROL VACCINIA RECOMBINANT EXPRESSING ENVELOPE GLYCOPROTEIN OF HIV.

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31 JUL 1990

WHAT IS CLAIMED IS:

1. A recombinant vaccinia virus selected from the group consisting of V(C-M-E-NS1-NS2_A)V(C-M-E), V(E), V(RSV G-E), V(FLU HA-E), and V(NS1-NS2_A), wherein the viral proteins are obtained from a flavivirus.

2. A recombinant vaccinia virus consisting essentially of the coding sequence for C, M, E, NS1, and NS2_A proteins, wherein the viral proteins are obtained from a flavivirus and 85 percent of the carboxyl terminal sequence of NS2_A protein is deleted.

3. The virus of claim 1 wherein said flavivirus is a dengue type 4 virus.

4. The virus of claim 2 wherein said flavivirus is a dengue type 4 virus.

5. The virus of claim 1, wherein said flavivirus is a Japanese B or tick-borne encephalitis virus.

6. The virus of claim 2, wherein said flavivirus is a Japanese B or tick-borne encephalitis virus.

7. A pharmaceutical composition, comprising an immunogenic amount of the virus of claim 1 in a pharmaceutically acceptable carrier.

8. A pharmaceutical composition, comprising an immunogenic amount of the virus of claim 2 in a pharmaceutically acceptable carrier.

9. A method for inducing protective immunity against a flavivirus disease, comprising inoculating a host acceptable to flavivirus with an immunogenic amount of the virus of claim 1 in at least a single dose.

10. A method for inducing protective immunity against a flavivirus disease, comprising inoculating a host acceptable to a flavivirus with an immunogenic amount of the virus of claim 2 in at least a single dose.

SUBSTITUTE SHEET

SUBSTITUTE SHEET
IPEA/US

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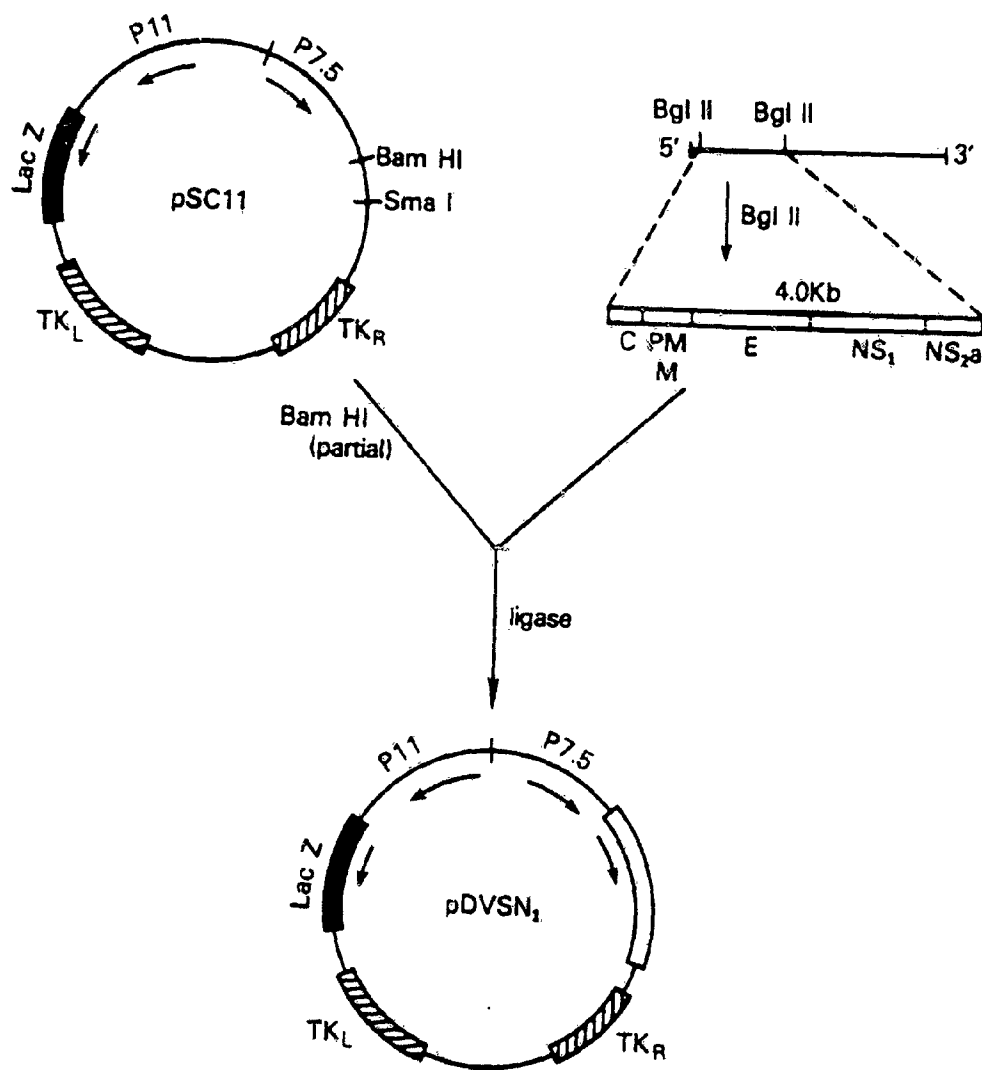
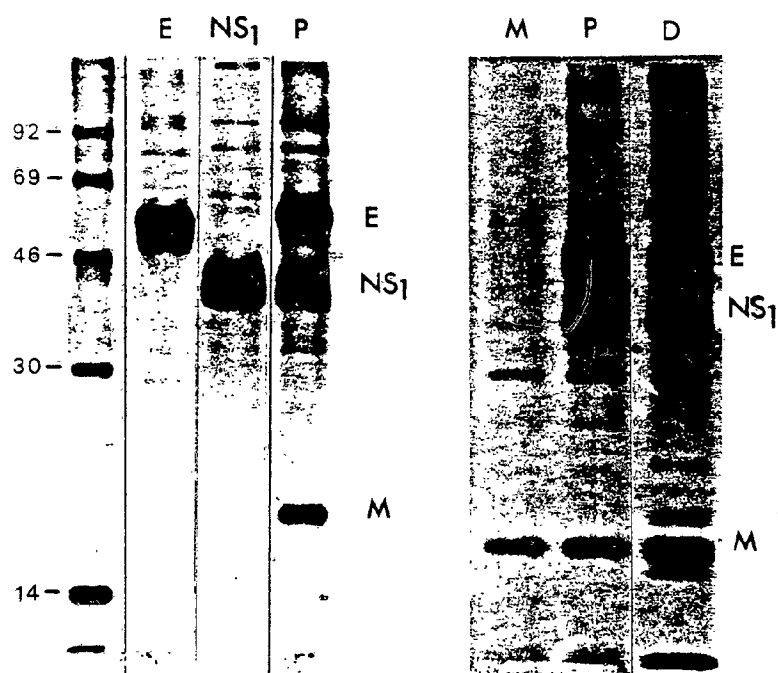


FIG.1

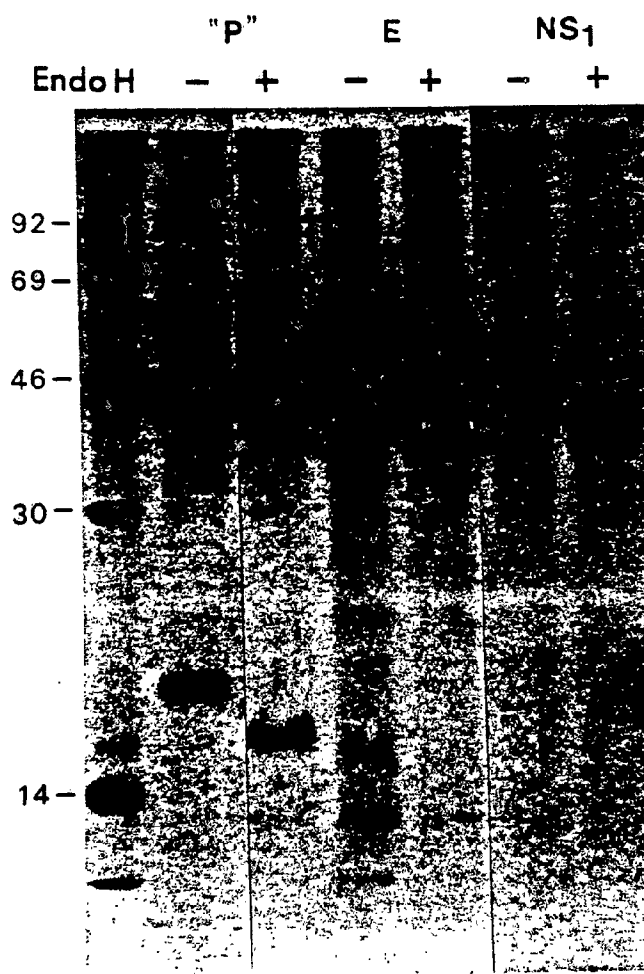
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FIG.2A



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FIG. 2B



INTERNATIONAL SEARCH REPORT

International Application No. PCT/US89/03589

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶

According to International Patent Classification (IPC) or to both National Classification and IPC
IPC(4): A61K 39/12; C12N 15/00; C12N 07/00

U.S. Cl.: 424/89; 435/172.3; 435/235; 435/320

II. FIELDS SEARCHED

Minimum Documentation Searched ⁷

Classification System	Classification Symbols
U.S.	435/172.3, 235, 320 935/6, 22, 59, 65 424/89

Documentation Searched other than Minimum Documentation
to the extent that such documents are included in the fields searched ⁸

Chemical Abstract Data Base (CA) 1967-1989; Biosis Data Base
1969-1989 Keywords: Flavivirus, vaccinia, dengue, type 4,
vaccine, Japanese B encephalitis, tick-borne; immun?

III. DOCUMENTS CONSIDERED TO BE RELEVANT ⁹

Category [*]	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X Y	JOURNAL OF VIROLOGY, Volume 61, issued 1987, December (B. ZHAO ET AL.), "Expression of dengue virus structural proteins and nonstructural protein NS1 by a recombinant vaccinia virus", See pages 4019-4022, see particularly the abstract and page 4019.	1-2 and 4-6 3
Y	JOURNAL OF VIROLOGY, Volume 62, issued 1988, August (Y. ZHANG ET AL.), "Immunization of mice with dengue struc- tural proteins and nonstructural protein NS1 expressed by baculovirus recombinant induces resistance to dengue virus encephalitis," See pages 3027-3031, See particularly the abstract.	1-6

* Special categories of cited documents: ¹⁰

"A" document defining the general state of the art which is not
considered to be of particular relevance

"E" earlier document but published on or after the international
filing date

"L" document which may throw doubts on priority claim(s) or
which is cited to establish the publication date of another
citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or
other means

"P" document published prior to the international filing date but
later than the priority date claimed

"T" later document published after the international filing date
or priority date and not in conflict with the application but
cited to understand the principle or theory underlying the
invention

"X" document of particular relevance: the claimed invention
cannot be considered novel or cannot be considered to
involve an inventive step

"Y" document of particular relevance: the claimed invention
cannot be considered to involve an inventive step when the
document is combined with one or more other such docu-
ments, such combination being obvious to a person skilled
in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

Date of Mailing of this International Search Report

12 October 1989

02 NOV 1989

International Searching Authority

Signature of Authorized Officer

ISA/US

Richard C. Peet

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	CHEMICAL ABSTRACTS, Volume 108, no. 7, issued 1988, February, E. Mackow et al. "The nucleotide sequence of dengue type 4 virus: analysis of genes coding for nonstructural proteins", See page 50123, column 2, the abstract no. 50120h, Virology, 1987, 159(2), 217-228(Eng.)	1-6
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V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE ¹

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers _____, because they relate to subject matter ¹² not required to be searched by this Authority, namely:

2. ☐ Claim numbers _____, because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out ¹³, specifically:

3. ☐ Claim numbers _____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING ²

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.
2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:
3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:
4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the international Searching Authority did not invite payment of any additional fee.

Remark on Protest

- ☐ The additional search fees were accompanied by applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	<u>CHEMICAL ABSTRACTS</u> , Volume 107, no. 7, issued 1987, August, P.W. Mason et al., "Expression of Japanese encephalitis virus antigens in Escherichia coli", See page 222, column 1, the abstract no. 53407X, Virology, 1987, 158 (2): 361-72(Eng.)	1-6
Y	<u>CHEMICAL ABSTRACTS</u> , Volume 107, no. 22, issued 1987, November, Research Foundation for Microbial Diseases, Osaka University, "Flavivirus antigen produced by recombinant DNA technology", See page 301, column 2, the abstract no. 205 157f, Belg. BE 905,815 (Eng).	1-6
Y	<u>CHEMICAL ABSTRACTS</u> , Volume 105, no. 23, issued 1986, December, C.J. Hai et al., "Cloning full-length DNA sequences of the dengue virus genome for use in elucidating pathogenesis and development of immunoprophylaxis", see page 157, column 1, the abstract no. 204133s, Vaccines 86, New Approaches Immun., 1986, 393-9 (Eng.)	1-6
Y	<u>CHEMICAL ABSTRACTS</u> , Volume 106, no. 7, issued 1987, February, A.G. Pletnev et al., "Nucleotide sequence of the genome region of the tick-borne encephalitis virus coding for structural proteins of virion", pages 44713, column 2, the abstract no. 44713w, Bioorg. Khim., 1986, 12/91, 1189-202 (Russ.	1-6